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Enzyme-Based Detection of Chlorinated Hydrocarbons in Water

JRB Associates, Inc., McLean, VA

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ENZYME-BASED DETECTION OF CHLORINATED HYDROCARBONS IN WATER

by

Barbara H. Offenhartz
Janet L. Lefko
B & M Technological Services, Inc.
Cambridge, MA 02142

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Project Officer

Michael D. Royer
Releases Control Branch
Hazardous Waste Engineering Research Laboratory
Edison, NJ 08837

HAZARDOUS WASTE ENGINEERING RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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16. ABSTRACT

An enzyme-based approach for detecting hazardous levels of high molecular weight chlorinated hydrocarbons in natural waters has been explored. An extensive review of the literature indicated that the enzymes, lactate dehydrogenase, carbonic anhydrase, hexokinase, phosphorylase and an ATPase are suitable for field method development. Lactate dehydrogenase (LDH) was chosen to develop a method that promises rapid, reliable and cost-effective detection. The LDH catalyzed reaction

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \longrightarrow \text{lactate} + \text{NAD}^+$$

is used to detect chlorinated hydrocarbons, which reduce the rate of the reaction by reversibly inhibiting the enzyme. The analysis uses pH detection and takes 5 minutes. So far, the LDH method has been used to detect aldrin, toxaphene, DDT, PCBs, pentachlorophenol and 2,4,5,-T at the parts per million level. A review of the literature suggests that most organochlorine pesticides, PCBs, polychlorinated phenols and chlorophenoxy derivatives can be detected by the LDH method. Preliminary investigations of potential interferants suggest that the LDH method selectively detects chlorinated hydrocarbons. Work was begun on immobilizing the enzyme for incorporation in water monitor and field detector designs. The LDH method shows potential for use in field devices and for processing extracts of soils and wastes, as well as water samples. These method adaptations are recommended for future work.

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FOREWORD

Today's rapidly developing and changing technologies and industrial products and practices frequently carry with them the increased generation of solid and hazardous wastes. These materials, if improperly dealt with, can threaten both public health and the environment. Abandoned waste sites and accidental releases of toxic and hazardous substances to the environment also have important environmental and public health implications. The Hazardous Waste Engineering Research Laboratory assists in providing an authoritative and defensible engineering basis for assessing and solving these problems. Its products support the policies, programs and regulations of the Environmental Protection Agency, the permitting and other responsibilities of State and local governments and the needs of both large and small businesses in handling their wastes responsibly and economically.

This report describes the development and limited testing of an enzyme-based detector that can detect at least five chlorinated hydrocarbons at concentrations of 10^{-4} to 10^{-6} in a 20 percent methanol/water solution. After screening numerous enzymes, lactate dehydrogenase (LDH) was selected for the detection system. Based on literature reviews, it is thought that the detector also will be sensitive to other high molecular weight chlorinated hydrocarbons. Phenol test data and analysis of the literature indicate that interference by nonchlorinated analogs will not occur. The simplicity and rapidity of the method indicate promise for field applications. The inhibition of the LDH by chlorinated hydrocarbons can be monitored either by pH or potentiometric changes in the reaction mixture; this provides flexibility that also enhances the potential of the detection method for field use.

For further information, please contact the Land Pollution Control Division of the Hazardous Waste Engineering Research Laboratory.

David G. Stephan, Director
Hazardous Waste Engineering Research Laboratory

ABSTRACT

This study explores a new approach for detecting hazardous levels of high molecular weight chlorinated hydrocarbons based on enzyme-catalyzed reactions. The lactate dehydrogenase (LDH) catalyzed reaction

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{[inhibitor]}]{\text{LDH}} \text{lactate} + \text{NAD}^+$$

is used to detect the presence of chlorinated hydrocarbons, which reduce the rate of the reaction by reversibly inhibiting the enzyme. Detection is straightforward, since the reaction produces a significant pH change. An analysis takes 5 minutes. Inhibition screening experiments show that the LDH method can detect aldrin, toxaphene, DDT, polychlorinated biphenyls (PCBs), and polychlorinated phenols at comparable levels of toxicity at parts per million (ppm) levels. A preliminary study of potential interferences is reported, as well as the results of an extensive literature search for suitable enzymes. In addition to LDH, four other commercially available enzymes were found to be potentially suitable: carbonic anhydrase, hexokinase, phosphorylase b, and an ATPase. LDH was chosen on the basis of a trade-off between sensitivity to inhibition and suitability for commercial method development.

The bench-top method studied in the laboratory appears readily adaptable to water-quality monitoring systems and pocketable field sensors. The development is patterned on the cholinesterase antagonist monitors (CAMs), developed under Environmental Protection Agency (EPA) sponsorship, which use the cholinesterase enzyme to detect organophosphate and carbamate pesticides. Work was begun on developing immobilized enzyme preparations suitable for use in this type of device.

Since the LDH enzyme is stable in a variety of water/organic solvent mixtures, including methanol, ethanol, and acetone, the method has potential for processing extracts of soils and organic wastes as well as water samples. In the LDH method reported the inhibitors were solubilized in a 20 percent methanol solution.

This report was submitted by B & M Technological Services, Inc. in fulfillment of Subcontract No. 2-817-33-956-05-5 with JRB Associates, Inc., under Contract No. 68-03-3113 with the U.S. Environmental Protection Agency. The report covers the period October 5, 1983 to July 31, 1983. Work was completed as of March 31, 1984.

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ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

ADP	--	adenosine diphosphate
ATP	--	adenosine triphosphate
ATPase	--	adenosine triphosphatase
CAM	--	cholinesterase antagonist monitor
ClCH	--	chlorinated hydrocarbon
DDD	--	2,2-Bis(p-chlorophenyl)-1,1-dichloroethane
DDT	--	2,2-Bis(p-chlorophenyl)-1,1,1-trichloroethane
EC-	--	Enzyme Control Commission enzyme number
IU	--	international unit of enzyme activity
LDH	--	lactate dehydrogenase
M	--	moles per liter
mg/l	--	milligrams per liter
mM	--	millimoles per liter
N	--	equivalents per liter
NAD^+	--	oxidized form of the cofactor nicotinamide adenine dinucleotide
NADH	--	reduced form of NAD^+
ng/l	--	nanograms per liter
nm	--	nanometers
P_i	--	inorganic phosphate
PCBs	--	polychlorinated biphenyls
ppm	--	parts per million
v_i	--	initial reaction velocity
W-A U	--	Wilbur Anderson unit of carbonic anhydrase activity
µg/l	--	micrograms per liter
µM	--	micromoles per liter

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We wish to acknowledge the contributions of the late Dr. Louis H. Goodson, whose pioneering work in the development of the CAM series of water monitors established enzyme-based detection systems as effective monitors of hazardous substances in the environment, and whose study on adapting other enzymes to CAM was the starting point for the work summarized in this report.

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SECTION 1

INTRODUCTION

A research and development program was undertaken to develop an enzyme-based method capable of detecting chlorinated hydrocarbons in water at hazardous levels and suitable for adaptation to field devices, such as automated water monitors and pocketable field detectors.

The design approach is patterned on the enzyme-based CAM (cholinesterase antagonist monitor) water quality monitors developed by Midwest Research Institute for the Environmental Protection Agency, Office of Research and Development (5, 6, 7). CAM water monitors detect hazardous levels of organophosphate and carbamate pesticides by their effect on the rate of a cholinesterase-catalyzed reaction. Design features common to CAM monitors are: an immobilized enzyme preparation, electrometric detection of reaction products, and an automated sampling cycle with automated sample handling. The field monitor, CAM-4, is an effective, cost-efficient field device (5, 18).

So far CAM monitors have been limited to incorporating the enzyme cholinesterase, inhibited by phosphate and carbamate pesticides. An earlier research effort to extend CAM's usefulness to detecting chlorinated hydrocarbons and other hazardous substances failed to incorporate new enzymes, but recommended carbonic anhydrase and hexokinase for further study (4).

This report summarizes the results of an extensive literature search and laboratory investigations undertaken to: 1) identify additional enzymes that have the potential to detect and monitor chlorinated hydrocarbons; 2) select the most promising enzyme for method development; and 3) develop a bench-top analytical method that is simple and reliable enough to warrant adaptation to field devices.

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SECTION 2

CONCLUSIONS

Investigations performed in the course of method research and development permit the following conclusions to be drawn.

The practical prerequisite of considering only commercially-available enzymes limited the work to developing methods for detecting dissolved high molecular weight chlorinated hydrocarbons.

Lactate dehydrogenase (LDH) is the best candidate enzyme identified, based on pollutant inhibition characteristics and potential simplicity of method design.

Hexokinase, carbonic anhydrase, phosphorylase, and ATPase are potentially useful for future method development.

The simple bench-top method developed, based on pollutant inhibition of an LDH-catalyzed reaction, has potential for adaptation to field detectors and CAM-type monitor designs.

The advantages of this method are: 1) analyses are rapid (5 minutes); 2) pH detection promises low-skill, reliable field performance; 3) preliminary tests suggest that most classes of high molecular weight chlorinated hydrocarbons can be detected at hazardous levels with minimal interference from other hydrocarbons, heavy metals and cyanide; 4) the enzyme's tolerance of organic solvents, such as acetone, dimethyl sulfoxide, methanol and ethanol, suggests that the method can be adapted to processing organic solvent extracts of soil samples and organic wastes, as well as water samples; and 5) cost-effective reuse of immobilized enzyme is expected since enzyme inhibition is reversible.

The disadvantages are that the method does not detect low molecular weight chlorinated hydrocarbons, such as carbon tetrachloride and trichloroethylene. In addition, the capability to assess water quality is limited by method sensitivity. The method detection limits obtained so far (500 µg/l) are below lethal levels for many aquatic species, but are much higher than upper limits recommended to protect aquatic life (1-3 µg/l) and human health (< 1 ng/l)*. Also, without modifications, the LDH method will

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*Notice of Water Quality Criteria Documents, 45 Fed. Reg., Appendix A, Summary of Water Quality Criteria, 79,324-79,341 (1980).

not detect organochlorine insecticides and polychlorinated biphenyls (PCBs) whose solubility in water is below the parts per million (ppm) level. Potentially detectable levels of these substances are bound to particulates suspended in water samples and must be solubilized prior to analysis.

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SECTION 3

RECOMMENDATIONS

The LDH bench-top method developed is a simple and effective approach to detecting high molecular weight chlorinated hydrocarbons at hazardous levels in water. Field device adaptation also looks promising, but enzyme immobilization methods require further development.

Field Sensors

The optimization of the LDH method and its adaptation to a pocketable field detector design is recommended. An enzyme ticket with visual color change detection and a reusable enzyme cartridge device using pH detection are potentially reliable and cost-effective design approaches. This design effort should include developing the capability to process extracts of soils and waste oils (the primary locus of PCB contamination).

Water Monitors

The adaptation of the LDH method to a continuous flow system is feasible and is recommended for attaining automated sample processing capability. The processing of water samples requires solubilizing particulate-bound chlorinated hydrocarbons. A recommended approach is to mix water samples with the ethanol solvent of the enzymatic reaction mixture prior to analysis. A sample pretreatment step of this type can be readily incorporated in the water monitor design. In addition, pretreatment steps should be explored that concentrate sample pollutants. The increase in sensitivity would enhance the practical application potential of water monitor designs.

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SECTION 4

METHOD DEVELOPMENT APPROACH

The objective of the effort reported was to identify enzymes and develop an enzyme-based method that could be incorporated in automated water monitors or other field detectors of chlorinated hydrocarbons. Potential adaptation to CAM-4 was particularly desirable to enhance its field monitoring capabilities.

The research and development program proceeded through the following steps:

Select Candidate Enzyme

(1) Search the literature to identify commercially-available enzymes that:

* have demonstrated inhibition toward one or more classes of chlorinated hydrocarbons; or
* have functionally related enzymes with demonstrated inhibition; or
* are implicated in biochemical mechanisms of chlorinated hydrocarbon toxicity.

(2) Interpret the above information using reaction mechanisms and appropriate structural information to select four to six promising enzymes.

(3) Select an enzyme with the best trade-off among the following characteristics:

* Enzyme inhibition indicates sensitivity and broad selectivity toward two or more classes of chlorinated hydrocarbons;
* Enzyme-catalyzed reaction can be monitored electrochemically (CAM or pH); and
* Immobilized enzyme preparations are robust.

(4) Laboratory test the enzymes selected to supplement published inhibition data.

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Adapt Enzyme Inhibition Method to Electrochemical Detection

Investigate continuity of method characteristics and demonstrate field adaptability. Perform appropriate laboratory studies including investigations of enzyme stability, enzyme inhibition characteristics, and potential method interferences.

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Design Review

Identify the field method or field device to be developed in consultation with the technical staff of the Municipal Environmental Research Laboratory, Edison, New Jersey.

Prepare and Study Immobilized Enzyme Preparations

Select an enzyme immobilization procedure appropriate for field device development. Perform laboratory investigations focusing on:

* Preparation of immobilized enzyme;
* Optimization of preparation with respect to enzyme activity, retention, stability and recyclability; and
* Study of inhibition characteristics of immobilized enzyme.

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SECTION 5

ENZYME SELECTION

A comprehensive literature review was conducted to select enzymes suitable for incorporation in chlorinated hydrocarbon water monitors and field sensors. Standard references and the computerized information retrieval resources of DIALOG Information Services, Inc., Defense Technical Information Center (DTIC), and National Technical Information Service (NTIS) were used to identify pertinent information to aid the selection process. The biomechanism of toxicity of chlorinated hydrocarbons influenced the structure of the review and its degree of success.

Chlorinated hydrocarbons change cell metabolism by interacting with enzymes and other macromolecules and by affecting membrane permeability (26). Of these modes of action, enzyme inhibition and induction can serve as detection schemes for chlorinated hydrocarbons (ClHC) where present as environmental pollutants:

Inhibition:	$X \xrightarrow[[ClHC]]{Enzyme} Y$	monitor decrease in reaction rate due to ClHC
Induction:	$ClHC \xrightarrow{Enzyme} Z$	measure Z

Both types of enzyme detectors depend on the enzyme's specificity for the pollutants and catalytic ability to provide selective, rapid measurements. Y (or X) and Z must be readily detectable by conventional means. An induction detector is reusable, conserving costly enzyme. An enzymatic detector operating on the inhibition principle is reusable if the inhibition is competitive, that is, if it can be reversed by high concentrations of the substrate X.

Inhibition by alteration of enzyme shape (allosterism) or competition with a cofactor such as NADH is reserved for the bulkier chlorinated hydrocarbons, such as those listed in Table 1. The enzyme inhibition schemes developed in this effort and the investigations of a prior research effort (4) focus on the detection of the classes of chlorinated hydrocarbons identified in Table 1 below.

Pollutants like carbon tetrachloride and trichloroethylene can only be detected with an induction device. The induction approach was beyond the

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TABLE 1. CHLORINATED HYDROCARBON TARGET COMPOUNDS

Cyclodiene Insecticides	Polychlorinated Phenols
Aldrin	Pentachlorophenol
Dieldrin	2,4,5-Trichlorophenol
Endrin	
Chlordane	Polychlorinated Biphenyls
Heptachlor	Aroclor-1242
Toxaphene	Aroclor-1245
Chlorinated Ethane Derivatives	**Miscellaneous Chlorinated Hydrocarbons**
DDT	Endosulfan
DDD	Diclone
Methoxychlor	Lindane

Chlorophenoxy Derivatives
2,4,5-T butyl esters
2-(2,4,5-trichlorophenoxy) proprionic acid

resources of this study, since promising induction enzymes are not commercially available. Enzyme extraction, purification and stabilization are the subjects of current research. (Note: Refer to EPA Research Grant No. R80532, G. Guilbault.)

Selection criteria were developed to facilitate the literature search for promising enzymes and to provide a scheme for ranking the enzymes identified. Sensitivity to inhibition by a broad spectrum of the target compounds of Table 1 and insensitivity to potential interferants were of primary importance. Practical considerations dictated that only commercially available enzymes be considered. Since enzymes with the same function but slight differences in protein structure (isozymes) can differ in sensitivity to inhibition, it was important that an isozyme with optimum sensitivity to chlorinated hydrocarbons be commercially available.

To facilitate method development, it was desirable to work only with enzymes whose physico-chemical characteristics were well documented and whose reaction mechanisms and molecular structure were well established. Field method characteristics of reliability, ease of use, and low cost were addressed by pursuing electrometric detection of enzymatic reaction products and a reusable, stable, immobilized enzyme preparation. Adaptability of detection schemes to the CAM-4 field monitor was desirable to extend its use as an enzyme-based monitor of organophosphate and carbamate pesticides to the detection of chlorinated hydrocarbons.

The literature review identified five enzymes suitable for method development. Table 2 lists the enzymes and their qualifications for method development. Bibliographies and more detailed information on inhibition

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TABLE 2. RANKING OF CANDIDATE ENZYMES

SELECTION CRITERIA	Lactate Dehydrogenase EC-1.1.1.27*	Carbonic Anhydrase EC-4.2.1.1*	Hexokinase EC-2.7.1.1*	Phosphorylase b EC-2.4.1.1*	ATPase EC-3.6.1.3*
Specificity and Sensitivity of Inhibition by Target Compounds	+**	+	+	+**	+**
Commercial Availability of Isozyme with Optimum Inhibition	+	+	+	+	+**
Documentation of Enzyme Characteristics	+	+	+	+	-
Electrometric Detection Scheme	++	+	-	-	-
CAM Hardware Adaptability	(+)	-	-	-	-
Immobilized Enzyme Characteristics	+	-	+	n.i.***	n.i.***
Reversibility of Inhibition	+**	-	-	-**	-**

* Enzyme Control Commission number
** Published information incomplete; characteristics inferred in part from information on enzyme structure and reaction mechanisms.
*** n.i. = no information found in literature search.

Range of acceptability: Exceptional ++, +, (+), - Unacceptable

characteristics, commercial preparations, assay procedures, enzyme structure and immobilization techniques are included in Appendix A.

Only two enzymes have been screened for inhibition by chlorinated hydrocarbons in each of the classes shown in Table 1: carbonic anhydrase (4, 15) and hexokinase (4, 21). Carbonic anhydrase and hexokinase are inhibited by most of the target compounds listed. Inhibition screening is incomplete for the enzymes phosphorylase (16, 25) and lactate dehydrogenase (9-11, 17). The inhibition characteristics of the commercial preparation of ATPase are inferred from data on structurally similar proteins (1, 2, 22). Even with incomplete information on inhibition characteristics, all enzymes have been shown to be inhibited by chlorinated hydrocarbons of two or more classes. Comparable detection thresholds are within a power of ten and at the ppm level.

Simplicity and cost of reaction schemes thus became a primary factor for choosing the best enzyme for method development. Only the LDH reaction can be monitored directly by electrometric detection, exhibits reversible inhibition, and shows potential for adapation to CAM-4. The carbonic anhydrase reaction is complicated by a competing reaction which makes pH-based detection unreliable (4). The reactions for hexokinase, phosphorylase b and ATPase require coupling with additional enzymatic reactions to permit electrochemical detection. LDH was chosen for method development. The LDH-based detection scheme and features important to method development are discussed below.

LDH catalyzes the interconversion of pyruvate and lactate in the presence of the cofactor nicotinamide adenine dinucleotide ($NADH/NAD^+$). The LDH isozyme, M_4, employed in method development, is the most sensitive to organochlorine pesticides which reduce the rate of the reaction:

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow[[\text{inhibitor}]]{\text{LDH}(M_4)} \text{lactate} + NAD^+$$

So far, only the effects of the pesticides dieldrin, aldrin, endrin, chlordane, mirex, kepone and DDT have been investigated (9-11, 17). Pesticide inhibition of LDH occurs at the ppm level and can be reversed by excess pyruvate or NADH. A similar effect is expected for chlorophenoxy derivatives, polychlorinated phenols and PCBs. This expectation is based on the well-established molecular structure and reaction mechanism of LDH isozymes (13). Experimental confirmation is presented in the inhibition studies described in Section 6, which also includes a review of the extensive literature on inorganic and organic substances known to affect LDH reaction rates. Several of these substances may interfere in the detection of chlorinated hydrocarbons in some environmental samples. Preliminary experimental investigations of potential interferants are also reported in Section 6. Based on inhibition characteristics alone, an LDH-based detection method can be expected to detect most of the high molecular weight hydrocarbons shown in Table 1 with few interferences. The inhibition

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mechanism (competitive) is well suited to the cost-effective reuse of immobilized enzyme.

The low water solubility (< 0.5 mg/l) of organochlorine pesticides and PCBs presents potential problems to the development of a simple and reliable enzyme detection method for water samples. When samples contain these pollutants in both dissolved and undissolved forms, the enzyme method should be able to detect both fractions. LDH in 20 percent methanol solutions detects both fractions, but according to different competing reaction schemes. The experimental work described in Sections 6 and 8 was aimed at optimizing the reversible enzyme reaction with dissolved pollutant, while suppressing the competing reaction with the undissolved sample fraction. An approach to solubilize undissolved pollutant is recommended for future application development.

The LDH-catalyzed reaction offers two options for monitoring the reaction rate electrometrically in a manner suitable for field use. Since H^+ is consumed in the reaction, pH changes can be measured directly. The work described in Section 7 develops this detection option. The redox couple NADH/NAD^+ can be monitored electrometrically, but requires additional reagents. Therefore, this detection option was not pursued. An NADH/NAD^+ detection scheme that has potential for adaptation to the CAM-4 hardware is discussed in Section 7.

Immobilizing the LDH enzyme promises to make LDH-based detection simple, reliable, cost-efficient and adaptable to field devices and diverse environmental samples. Several types of immobilization techniques have been applied successfully to LDH (8, 12, 14, 20, 23). The physico-chemical characteristics of the immobilized preparations are well documented. Insensitivity to heat, organic solvents and acidic pH is improved greatly by immobilizing the enzyme. Initial work on immobilizing LDH is reported in Section 8.

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SECTION 6

LDH INHIBITION STUDIES

The laboratory investigations were designed to demonstrate that an analytical method for detecting chlorinated hydrocarbons can be based on the pollutant inhibition of lactate dehydrogenase. The investigations consisted of the design of an appropriate inhibition test procedure; inhibition screening with representative test compounds and a test of the kinetic mechanism of inhibition; and a preliminary study of potential method interferences.

Enzyme Inhibition Assay

The starting point for the design of the inhibition screening test was the standard assay procedure for LDH described in Appendix B. The standard assay monitors the rate of disappearance of NADH during the course of the reaction

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[[\text{inhibitor}]]{\text{LDH}} \text{lactate} + \text{NAD}^+$$

by spectrophotometrically determining the absorption of the reaction mixture at the 340 nm peak of NADH as a function of reaction time.

The standard assay had to be modified to permit active solubilized enzyme to react with the dissolved chlorinated hydrocarbon test compounds (concentration range 10^{-4} M to 10^{-6} M). Inhibition effects, if present, are detectable by modified assays under these conditions.

Since organochlorine pesticides and PCBs are less soluble in water than 10^{-6} M, a different solvent was chosen to increase the solubility of the test compounds to the required level. The enzyme inhibition tests of the literature employ water/miscible organic solvent formulations containing 5 to 20 percent by volume of the organic solvent. The particular solvent formulation chosen was one that has the least adverse effect on enzyme activity and promotes the greatest increase in test compound solubility.

The solvents, dimethyl sulfoxide, ethanol, methanol and acetone, were tested with the chlorinated hydrocarbon test compounds identified in Table 3. Solubility limits were determined by visual inspection and by measuring spectrophotometric absorption in the 300 to 340 nm range.

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TABLE 3. CHLORINATED HYDROCARBON TEST COMPOUNDS

TEST COMPOUND	SOLUBILITY IN:* WATER	SOLUBILITY IN:* 20% METHANOL BUFFER	EPA RQ† CATEGORY	AMBIENT WATER CRITERIA** FRESH WATER	AMBIENT WATER CRITERIA** HUMAN HEALTH
CYCLODIENE INSECTICIDES					
Aldrin	●	⊖	X	3.0 µg/l	.74 ng/l
Dieldrin	●	⊖	X	2.5 µg/l	.76 ng/l
Endrin	●	⊖	X	0.18 µg/l	(1 µg/l)
Chlordane	●	⊖	X	2.4 µg/l	4.6 ng/l
Toxaphene	●	⊖	X	1.6 µg/l	7.1 ng/l
CHLORINATED ETHANE DERIVATIVES					
P,P' DDD	●	⊖	X	1.1 µg/l	.24 ng/l
P,P' DDT	●	⊖	X	1.1 µg/l	.24 ng/l
Methoxychlor	●	⊖	X	-	-
POLYCHLORINATED BIPHENYLS					
Aroclor 1242	0	⊖	A	2 µg/l	.79 ng/l
Aroclor 1254	0	⊖	A	2 µg/l	.79 ng/l
POLYCHLORINATED PHENOLS					
Pentachlorophenol	0	0	A	55 µg/l	(1 mg/l)
2,4,5-Trichlorophenol	0	0	A	970 µg/l	12 µg/l
CHLOROPHENOXY DERIVATIVES					
2,4,5-Trichlorophenoxy Acetic Acid	0	0	B	-	-
2-(2,4,5,-Trichlorophenoxy) Proprionic Acid	0	0	B	-	-
MISCELLANEOUS CHLORINATED HYDROCARBONS					
Dichlone	●	⊖	X	-	-
Lindane	0	0	X	-	-

* Solubility: ● < 10^{-6} M; 5×10^{-5} < ⊖ < 10^{-4} M; 0 > 10^{-4} M.
Conversion factor applicable: 1×10^{-5} M corresponds to 2-4 mg/l.
With the exception of dieldrin (85%), chlordane (95%) and toxaphene (Tech. grade), the purity of the compounds tested was 97% or better.

† Category X hazardous substances designated in 40 CFR 117.3 have a reportable quantity of 1 lb; for Category A, the reportable quantity is 10 lb; for Category B, the reportable quantity is 100 lb.

** Ambient Water Criteria: upper limits recommended by the EPA to protect aquatic life and human health. Notice of Water Quality Criteria Documents, 45 Fed. Reg., Appendix A, Summary of Water Quality Criteria, 79,324-79,341 (1980).

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Solvent effects on enzyme activity were investigated to determine the best trade-off between test compound solubility and enzyme activity. The results of the methanol study, covering the range 0 to 30 v/v% methanol/phosphate buffer mixtures, are summarized in Figure 1. Adding 10 v/v% and 20 v/v% methanol to the enzyme assay heightened LDH activity, a result which is consistent with George's (1969) finding that the short and long-term stability of LDH is enhanced in organic solvent formulations (3). The effect of methanol formulations on LDH activity suggests that methanol is a better solvent than ethanol as used in published LDH inhibition studies (10).

The optimal methanol concentration for enhancing LDH activity is about 20 v/v%, and a 20 v/v% methanol formulation was incorporated in the inhibition assay procedure detailed in Appendix B. The corresponding test compound solubilities are listed in Table 3.

Inhibition Screening

In Table 3 the target compounds of Table 1 are relisted with information pertinent to the planning and evaluation of the inhibition screening investigation. Chlorinated hydrocarbons are listed according to chemical classes whose members are expected to have similar inhibition effects for structural reasons. The EPA RQ (reportable quantity) classifications are an approximate guide to the relative toxicity of classes, X > A > B*. EPA recommended ambient water limits† establish targets for the desired sensitivities of water quality monitors. Solubilities in water and in the inhibition test reaction mixture provide a further classification scheme which is useful in the evaluation of inhibition screening data.

Inhibition tests of selected representative test compounds were performed according to the enzyme inhibition assay developed previously. Table 4 summarizes the results of more than 70 assays performed with seven test compounds at three different inhibitor concentrations. The inhibition results are consistent with the published literature where overlap of data exists, e.g., aldrin (9). LDH sensitivity to chlorinated hydrocarbons approximately parallels the relative toxicity of the compounds. The 2,4,5-T inhibition of 19 percent is marginally significant, and 2,4,5-T should be retested at higher concentrations. Lindane was not expected to effect LDH for structural reasons.

The "100**" values indicate complete inhibition of the enzyme and require further explanation. Lactate dehydrogenase, like all of the enzymes documented in Appendix A, reacts with dissolved as well as

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* Category X hazardous substances are the 21 organic compounds designated in 40 CFR 117.3 as having a reportable quantity of 1 lb; Category A substances require the reporting of 10 lb; Category B, 100 lb.

† Notice of Water Quality Criteria Documents, 45 Fed. Reg., Appendix A, Summary of Water Quality Criteria, 79,324-79,341 (1980).

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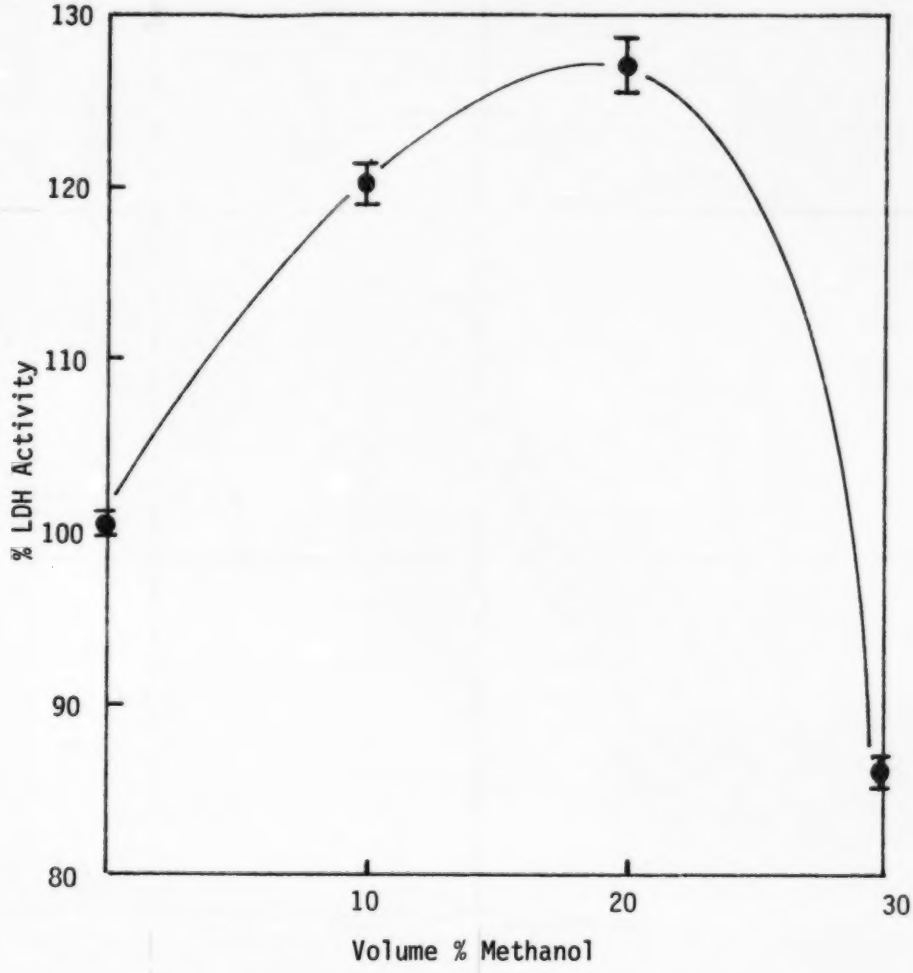

Figure 1. The effect of methanol on LDH activity.

Experimental conditions: [NADH] = 0.220 mM; [pyruvate] = 1.00 mM; 0.1 unit of LDH, M_4 isozyme in 0.1 M phosphate buffer containing 0 - 30% methanol by volume, pH 7.3 at 23°C. LDH activity was assayed by the initial rate of disappearance of NADH at 340 nm of a Hitachi 100-80 spectrophotometer.

TABLE 4. INHIBITION OF LACTATE DEHYDROGENASE

TEST COMPOUND	% INHIBITION* AT SEVERAL CONCENTRATIONS		
	10^{-4} M†	10^{-5} M	10^{-6} M
CYCLODIENE INSECTICIDES			
Aldrin	100**	58 ± 8	29 ± 3
Toxaphene	100**	40 ± 2	13 ± 4
CHLORINATED ETHANE DERIVATIVES			
DDT	100**	22 ± 3	6 ± 5
POLYCHLORINATED BIPHENYLS			
Aroclor 1242	100**	22 ± 12	0
POLYCHLORINATED PHENOLS			
Pentachlorophenol	74 ± 8	23 ± 6	0
CHLOROPHENOXY DERIVATIVES			
2,4,5-Trichlorophenoxy Acetic Acid	19 ± 7	7 ± 7	-
MISC. CHLORINATED HYDROCARBONS			
Lindane	0	0	-

* Percent inhibition was calculated by assigning 100 percent activity to a control containing no test compound.

† Conversion factor applicable: 1×10^{-5} M = 2-4 mg/l.

** Undissolved test compound present.

Experimental conditions: [NADH] = 0.220 mM; [pyruvate] = 1.00 mM; [test compound] = 0 (control), 1×10^{-6} M, 1×10^{-5} M or 1×10^{-4} M; 0.1 unit of LDH in 0.11 M phosphate-methanol buffer (80:20 v/v%), pH 7.3, 23°C. The addition of enzyme, pre-equilibrated in phosphate-methanol buffer, started the reaction. Enzyme activity was determined by the method of initial reaction rates. The disappearance of NADH was followed spectrophotometrically with a Hitachi Model 100-80 at 340 nm. The 300 to 340 nm background absorption was checked for the presence of undissolved reaction components.

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undissolved chlorinated hydrocarbons. The reactions are quite different. The reaction with undissolved inhibitor dominates, as illustrated by the 100** results.

Undissolved chlorinated hydrocarbons bind enzyme by coprecipitation, a process that reduces reaction rates by removing enzyme from solution (19). Only dissolved enzyme and dissolved chlorinated hydrocarbon participate in the reversible reaction that forms the basis of the enzymatic detection method to be developed.

Coprecipitation makes the detection method technique-dependent and prone to false highs when undissolved inhibitor is present. Both are undesirable features in a method intended for field use. Enzyme immobilization (Section 8) provides the opportunity to suppress coprecipitation and make detection simple and reliable.

The data of Table 4 could overestimate inhibition effects projected for field devices if undetected undissolved inhibitor were present at the lower concentrations tested ($< 10^{-4}$ M). This possibility was examined in the kinetic mechanism studies of Figures 2 and 3. The double reciprocal plots (Lineweaver-Burk) of the initial reaction velocity v_i versus the pyruvate concentration for Aroclor 1242 and pentachlorophenol are consistent with reversible inhibition only and consistent with the absence of undissolved inhibitor in these investigations.

Inhibition by Other Hazardous Substances

The capability to detect other chemicals, particularly at less than toxic levels, would detract from the usefulness of this method. A review of the LDH literature and the inhibition tests reported below provide a preliminary assessment of potential method selectivity.

Nonhalogenated aromatic pollutants, with the exception of phenols and amines, are even less soluble than their chlorinated analogs, so that interference is unlikely. Interference is not expected for structural reasons. Phenol was tested and exhibited no significant inhibition at concentrations less than 10^{-1} M. These results suggest that the LDH method may be quite specific for halogenated aromatics of appropriate ring structure, but further testing is required.

The organophosphate pesticide Diazinon and the carbamate Sevin inhibited LDH only at concentrations greater than 10^{-3} M. This indicates that the enzyme LDH, and the enzyme cholinesterase (of the CAM monitors) have complementary detection capabilities. The LDH method selectively detects high molecular weight chlorinated hydrocarbons, the CAM method, organophosphate and carbamate pesticides.

Cyanide tends to inhibit most enzymes that require metal ions for full expression of enzyme activity. LDH has no metal ion requirement (13).

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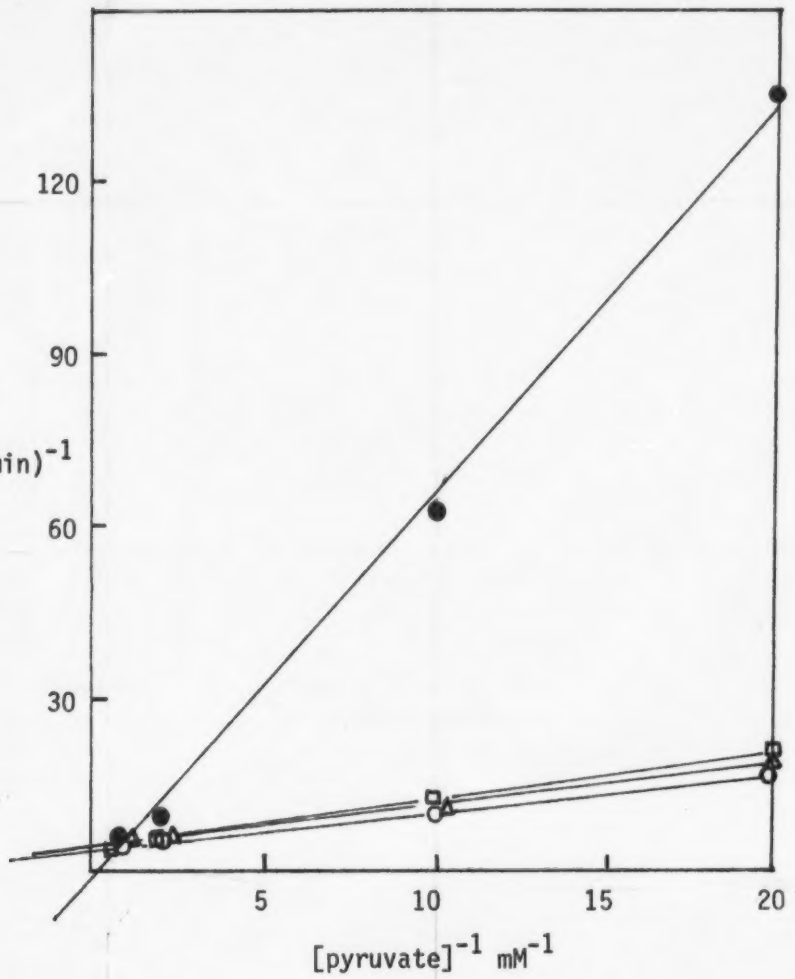

Figure 2. The effect of Aroclor 1242 on lactate dehydrogenase.

Lineweaver-Burk double reciprocal plot of the effect with respect to pyruvate. Experimental conditions: [NADH] = 0.220 mM; [pyruvate] = 0.050 - 1.00 mM; [Aroclor 1242] = (o) 0 mM, (△) 0.005 mM, (□) 0.010 mM, (●) 0.050 mM; 0.1 units LDH in 0.11 M phosphate-methanol buffer (80:20 v/v%) at pH 7.3, 23°C. Spectrophotometric determination of the initial rate of disappearance of NADH (v_i) with a Hitachi Model 100-80 at 340 nm.

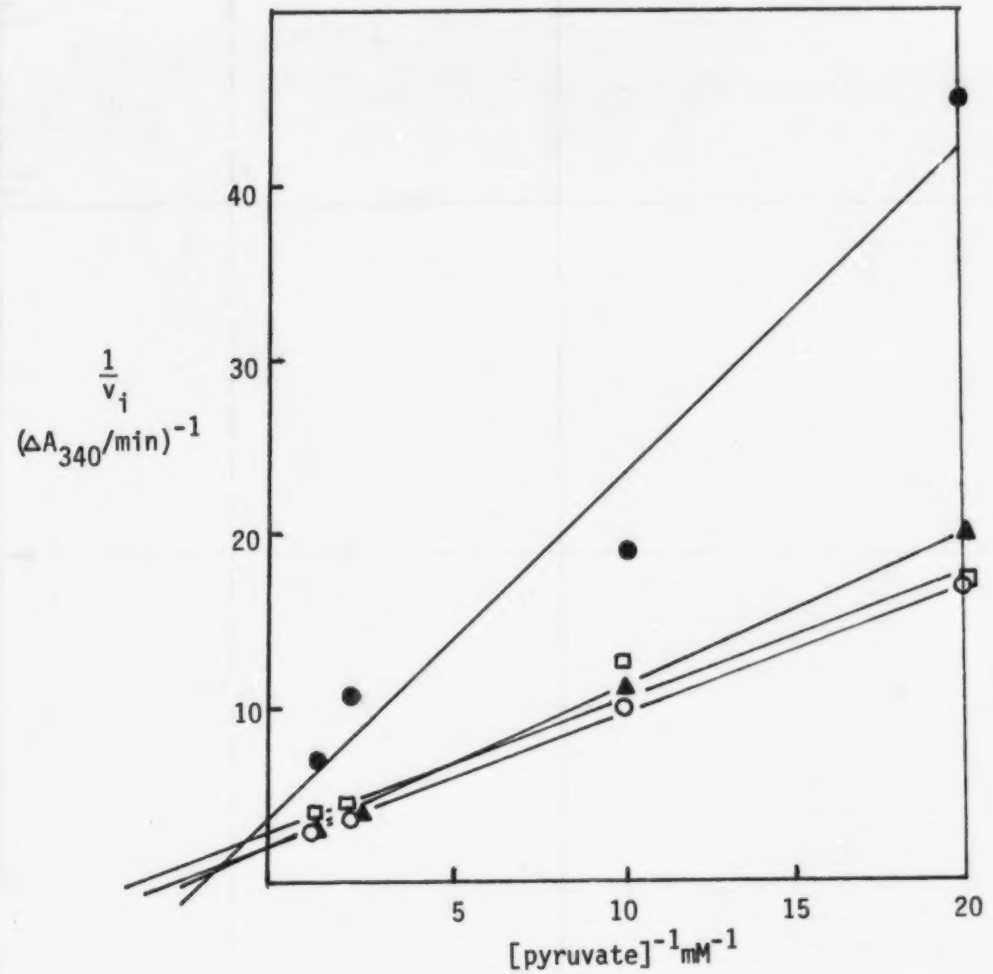

Figure 3. The effect of pentachlorophenol on lactate dehydrogenase.

Lineweaver-Burk double reciprocal plot of the effect with respect to pyruvate. Experimental conditions: [NADH] = 0.220 mM; [pyruvate] = 0.050 - 1.00 mM; [pentachlorophenol] = (o) 0 mM, (▲) 0.005 mM, (□) 0.010 mM, (●) 0.050 mM; 0.1 units LDH in 0.11 M phosphate-methanol buffer (80:20 v/v%) at pH 7.3, 23°C. The initial rate of disappearance of NADH (v_i) was determined spectrophotometrically with a Hitachi Model 100-80 at 340 nm.

Concentrations as high as 10^{-2} M cyanide were required to exhibit 50 percent inhibition. The LDH method is relatively insensitive to hazardous levels of cyanide.

Heavy metal ions, e.g., free Hg^{+2}, showed complete inhibition consistent with the presence of a cysteine (-SH) and a histidine group at the active site of LDH (13). The addition of a suitable chelating agent to reagent formulations will be able to suppress potential interference in environmental applications that contain high levels of heavy metal ions. The ability of chelating agents to reverse the inhibition of LDH with metal ions is well documented (13).

Alkylation of the essential histidine and cysteine can result in the slow, irreversible inhibition of the enzyme. The alkylation by α-halocarboxylic acids (halo = I, Br, Cl) has been investigated extensively (13). While these studies suggest that enzyme deactivation occurs too slowly to interfere in the timeframe of the 5-minute analysis cycle, an immobilized enzyme detector will require frequent replacement if alkylating agents are present in sample media.

In summary, the LDH inhibition studies of this report and the literature show that LDH is affected by a broad range of high molecular weight chlorinated hydrocarbons: organochlorine insecticides (9-11, 17), the PCB mixture Aroclor 1242, pentachlorophenol, and 2,4,5-trichlorophenoxy acetic acid. LDH appears quite selective toward chlorinated hydrocarbons. The analytical window is small, but can be fine-tuned by the choice of solvent, the enzyme activity, pyruvate and NADH concentrations, and the type of immobilized enzyme preparation developed. Detection thresholds are high ($>$ 0.5 mg/l) relative to the ambient water criteria of Table 3. This limits the practical application of water quality monitors incorporating an LDH-based detection method, unless samples are extracted and concentrated prior to analysis. Extraction/concentration procedures are recommended for future development.

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SECTION 7

LDH METHOD WITH pH DETECTION

The task of adapting the colorimetric enzyme assay to a detection method better suited to field conditions was addressed at the completion of the inhibition studies.

Electrometric detection methods are inherently more reliable than colorimetry, since natural water samples tend to have background absorption and to foul optical surfaces. Fouling of electrode surfaces is a less serious problem that can be addressed adequately by routine maintenance.

The LDH-catalyzed reaction

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{[inhibitor]}]{\text{LDH}} \text{lactate} + \text{NAD}^+$$

offers two options for electrometric detection: 1) the redox couple [NADH]/[NAD^+] can be monitored; or 2) since H^+ is consumed, the pH change can be measured directly by a pH meter.

The former is more easily adapted to the CAM hardware but requires additional reagents. Since NADH and NAD^+ are not well-behaved electroactive species, coupling with a redox mediator is required for electrometric detection. Bindschedler's Green has been used as the redox mediator for an amperometric determination of LDH activity (24). This detection approach has potential for adaptation to the CAM-4 hardware. The simpler pH detection option was chosen for method development.

In adapting the colorimetric enzyme inhibition assay to an assay with pH detection, a change of buffer, from phosphate to acetate, was required to maintain method sensitivity. The enzyme reaction of the colorimetric assay occurs in a neutral buffered environment containing an acid-base ratio designed to maintain the system at an optimal pH. This buffer system, by definition, resists change in pH. Decreasing the acid-base molar concentrations and/or increasing the acid-base ratio defines a buffer system that will reflect changes in pH.

Since the reaction consumes hydrogen ion, the pH of the reaction mixture should increase with time. The extent of the pH increase depends upon the buffering capacity of the reaction mixture. When the reaction mixture was titrated with hydroxide, addition of 10^{-4} M OH^- at a pH initially equal

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to 6 produced a 2-unit pH change rather than the 3-unit change expected in an unbuffered reaction mixture. The difference was traceable to the buffering effect of the commercial enzyme preparation. As shown in Figure 4, when the reaction mixture (primarily sodium acetate and pyruvate) is titrated in the absence of enzyme, a far sharper pH change is observed. (A sodium acetate buffer limits pH decrease, but has no effect on pH increase.) Nevertheless, as shown in Figure 5, the buffering effect of the enzyme preparation is not so great as to eliminate pH detection as a viable method. In the presence of high concentrations of a chlorinated hydrocarbon, the pH change would be zero; the pH change actually observed, in excess of 1 unit, clearly indicated the absence of inhibitors. The pH inhibition assay procedure is provided in Appendix B.

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We conclude from these results that a 1 mM acetate-methanol buffer (80:20 v/v%) with an initial pH of 6.7 to 7.0 supports a relatively robust detection scheme. At this pH and methanol concentration, the enzyme activity differs insignificantly from the enzyme activity of the colorimetric inhibition assay. Similar pollutant inhibition characteristics are expected, since the enzyme activity remains comparable (10). The signal-to-noise ratios of the colorimetric and pH detection schemes are comparable.

The solution formulation is well buffered against normal acidic waters. Moreover, acetate buffer strength can be increased to accommodate greater sample acidity. This formulation is not expected to handle waters more basic than pH 7, such as seawater or brackish water samples. In principle, different buffer formulations can be developed to accommodate these samples. Alternatively, an electrometric detection scheme of the redox couple NADH/NAD^+ could be developed.

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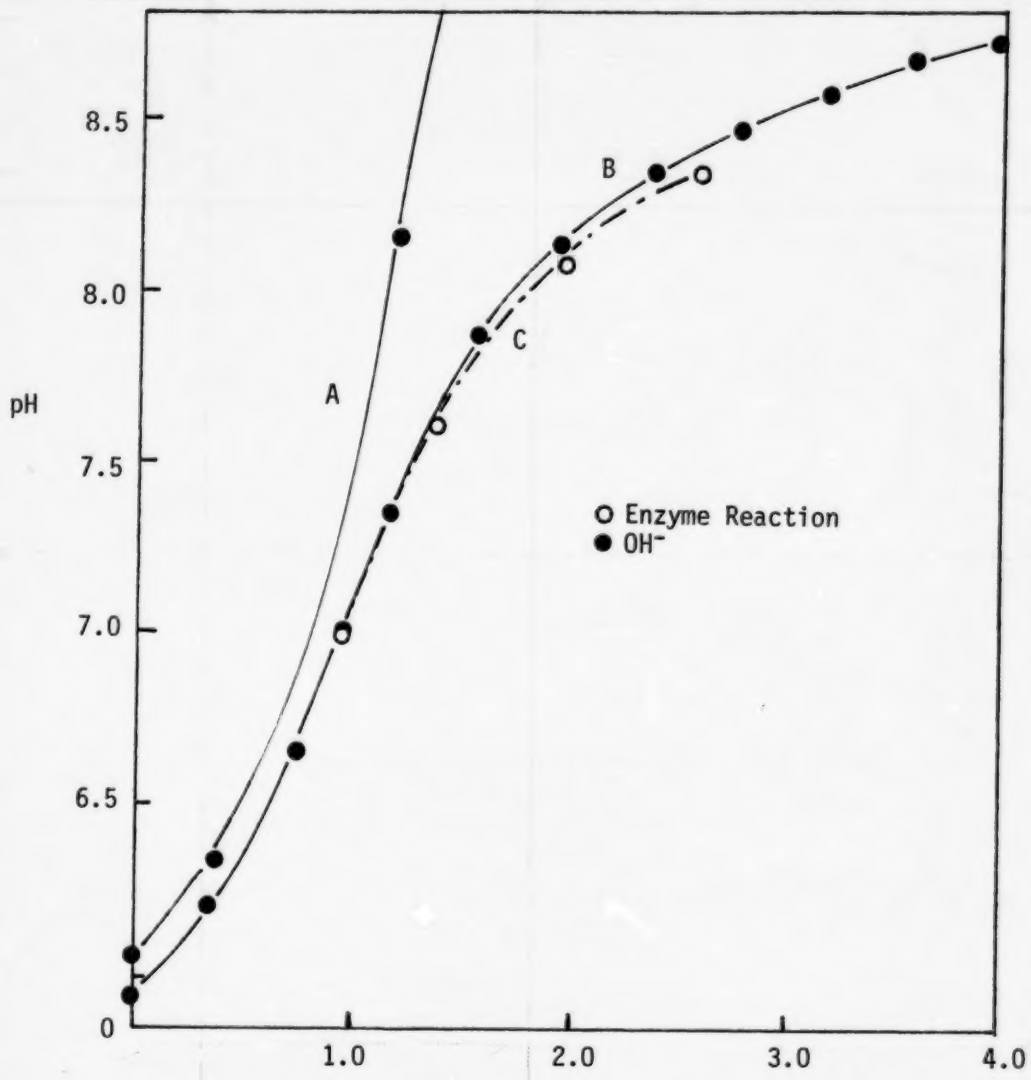

Figure 4. Titration of the LDH reaction mixture.

In titration A the components NADH and pyruvate in buffer are titrated with 0.1 N NaOH. In titration B the enzyme in buffer is titrated. In titration C the enzyme reaction consumes H^+ (initial pH = 7.0). The change in $[H^+]$ is calculated from the change in [NADH], determined spectrophotometrically at 340 nm; $\Delta[H^+] \equiv \Delta[NADH]$. Experimental conditions: [NADH] = 0.22 mM; [pyruvate] = 1.00 mM; 0.40 units LDH in 1 mM acetate buffer-methanol (80:20 v/v%); 23°C.

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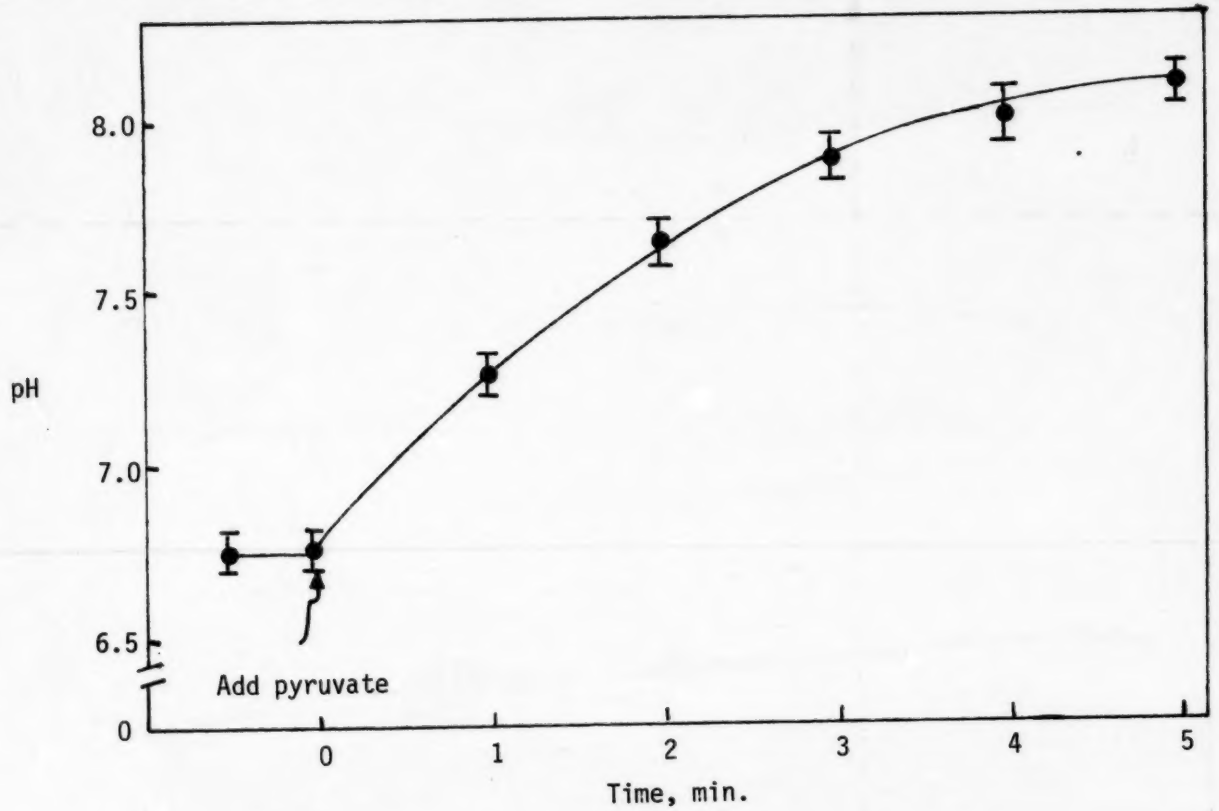

Figure 5. pH detection of the LDH catalyzed reaction.

Experimental conditions: [NADH] = 0.22 mM; [pyruvate] = 1.00 mM; 0.4 units LDH in 1 mM acetate buffer-methanol (80:20 v/v%); 23°C. The pH was measured with a Beckman Model 76 pH meter.

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SECTION 8

LDH IMMOBILIZATION

Immobilization of the LDH enzyme is a necessary step toward developing reliable, cost-efficient field methods. Many different types of immobilization techniques have been applied successfully to LDH. Several look quite promising for developing enzyme preparations suitable for continuous flow applications like the CAM monitors, and for field detector designs (8, 12, 14, 20, 23).

Bonding the enzyme to glass or organic polymer supports or entrapping the enzyme in a gel matrix improves enzymatic detection in several ways. The enzyme can be incorporated directly in the detector design. This eliminates time-consuming, technique-sensitive steps from the analytical procedure. Immobilization promotes cost-efficient reuse of the enzyme for multiple analyses. The reversible nature of LDH inhibition insures that enzyme activity can be regenerated after each analytical cycle. Immobilized LDH preparations have high enzyme activity, show excellent long-term stability and resist degradation by heat, organic solvents and acidic and basic media (8, 12, 14, 20, 23). The characteristics are superior to those exhibited by the free enzyme used in this work. Therefore, immobilized LDH preparations developed for field devices can be expected to require infrequent replacement and to have potential for processing organic extracts of soils, wastes and water samples.

Immobilizing LDH can alter the inherent sensitivity of the enzyme to inhibition by chlorinated hydrocarbons. The enzyme support changes the environment of the enzyme reaction and can restrict the diffusion of reactants toward the enzyme surface. Diffusion limitations can suppress coprecipitation of the enzyme with undissolved inhibitor, a desirable effect. Diffusion can also alter enzyme sensitivity to dissolved inhibitor. Each immobilization method must be investigated with care to insure that enzyme activity and sensitivity and selectivity for chlorinated hydrocarbons are maintained.

A polyacrylamide gel entrapment technique was investigated first, since LDH gel preparations are commercially available. If used instead of free enzyme in the LDH method, this adaptation could provide laboratories with a robust, bench-top method early in the course of device development, an application of interest to members of the project design review panel. Granular gel preparations can also be packed in columns suited to CAM-type instrumentation and reusable enzyme cartridge detectors.

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The enzyme immobilization studies focused on adapting established methods for LDH entrapment in a polyacrylamide gel matrix (12, 14, 23) and studying the characteristics of the dispersed LDH gels prepared. The methods developed for immobilizing the enzyme and assaying its activity are included in Appendix B.

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Among the characteristics of the LDH gels studied, only the inhibition data were disappointing. The LDH gels were less than one-tenth as sensitive to aldrin inhibition, for example, as the free enzyme previously studied. Preincubation with inhibitor was required to achieve full expression of the inhibition effect. While the LDH polyacrylamide gel preparation and procedure are not optimized as yet, a 10- to 100-fold increase in method sensitivity may be difficult to achieve.

The difference in inhibition characteristics of free enzyme and enzyme immobilized in a gel matrix is not surprising, since different reaction environments are involved, and diffusion becomes a factor. Among other LDH immobilization techniques, the covalent bonding methods (8) employing a spacer molecule between the enzyme and its support are best suited to reproducing the free enzyme environment in an immobilized enzyme preparation. These techniques are recommended for future method development.

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APPENDIX A

ENZYME FACT SHEETS

I. Lactic Dehydrogenase EC-1.1.1.27

Reaction Catalyzed: Pyruvate + NADH + H+ = Lactate + NAD+

Supplier: Sigma Chemical (L 5132 Type V-S, LDH-5 (M_4) Isoenzyme)

Biological Source: Rabbit muscle

Stability: Approx. 6-12 mos. at 5°C.

Activity: Approximately 800 IU/mg

Cost: $6.00/mg

Reported Inhibition by Aromatic Chlorinated Hydrocarbons:

Dieldrin, aldrin, endrin, chlordane, kepone and mirex

Assay Procedure:

Worthington Enzyme Manual (A. Decker, editor), Worthington Biochemical Corporation, Freehold, NJ, 1977, pp. 19 - 22.

Electrochemical Detection:

1. Monitor NAD+/NADH.
2. Measure pH.

References:

Inhibition and Molecular Structure:

1. Anderson, B.M., Kohler, S.T. and Young, R.W., "Interactions of Kepone with Rabbit Muscle Lactate Dehydrogenase," J. Agric. Food Chem., 26(1):130-133, 1978.

2. George, H., J. McMahen, K. Bowker, and M. Elliott, "Stabilization of Lactate and Malate Dehydrogenase by Organic Solvents," Biochem. Biophys. Acta. 191:466-468, 1969.

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Immobilization:

9. Guire, P., "Photochemical Immobilization of Enzymes and Other Biochemicals," Chapter 21, Methods in Enzymology, Vol. XLIV, (Klaus Mosback, editor), Academic Press, New York, 1974.

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II. Carbonic Anhydrase EC-4.2.1.1

Reaction Catalyzed: $H_2CO_3 = CO_2 + H_2O$

Supplier: Sigma Chemical (C 7500)

Biological Source: Bovine Erythrocytes

Stability: Estimated 1 yr. at 5°C.

Activity: 2,500 W-A U/mg

Cost: $9.80/50mg

Reported Inhibition by Aromatic Chlorinated Hydrocarbons:

Dieldrin, DDT, chlordane, 2,4,5-T, methoxychlor, toxaphene, pentachlorophenol, 2,4,5-trichlorophenol, etc.

Assay Procedure:

Wilbur, K.M. and N.G. Anderson, J. Biol. Chem., 176:147, 1948.

Electrochemical Detection:

Measure pH, but uncatalyzed hydrolysis competes.

References:

Inhibition and Molecular Structure:

1. Goodson, L.H. and V.J. Appleman, "Alternate Enzymes for Use in Cholinesterase Monitors (CAMs)," U.S. Environmental Protection Agency Publication No. EPA-600/2-80-083, 1980.

2. Kandell, M. and A.G. Gonnall, "Some Characteristics of Human, Bovine and Horse Carbonic Anhydrases Revealed by Inactivation Studies," J. Biol. Chem., 245(9):2444-2450, 1970.

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Immobilization:

1. Edman, P., B. Ekman, I. Sjoholm, "Immobilization of Proteins in Microspheres of Bio Degradable Poly Acryl Dextran," J. Pharm. Sci., 69(7):838-842, 1980.

2. Ekman, B., I. Sjoholm, "Improved Stability of Proteins Immobilized in Micro Particles Prepared by a Modified Emulsion Polymerization Technique," J. Pharm. Sci., 67(5):693-696, 1978.

3. Epton, R., M.E. Hobson, and G. Marr, "A Study of Carbonic Anhydrase Covalently Bound to a Poly Acryloyl Morpholine Network in Aqueous Organic Solvents," Biochem. Soc. Trans., 5(1):274-276, 1977.

4. Horowitz, R., and G.M. Whitesides, "Generalized Affinity Chromatography Enzyme Sulfonamide Conjugates Can Be Isolated by Adsorption on Immobilized Carbonic Anhydrase EC-4.2.1.1," J. Am. Chem. Soc., 100(14):4632-4633, 1978.

5. Ray, B., "Purification and Immobilization of Human Carbonic Anhydrase b by Using Poly Acrylamide Gel," Experientia, 33(11):1440, 1977.

III. Hexokinase EC-2.7.1.1

Reaction Catalyzed: ATP + D-hexose = ADP + D-hexose-6-phosphate

Supplier: Sigma Chemical (H 5375, Hexokinase type VI)

Biological Source: Yeast

Stability: Estimated 12 mos. at 5°C.

Activity: 40-50 IU/mg

Cost: $8.00/1000 U

Reported Inhibition by Aromatic Chlorinated Hydrocarbons:

Aldrin, chlordane, DDT, heptachlor, pentachlorophenol, trichlorophenol, 2,4,5-T acid, lindane

Assay Procedure:

Darrow R.A., and S.P. Colowick, Methods in Enzymology, Vol. V, (S.P. Colowick, and N.O. Kaplan, editors), New York: Academic Press, 1962, p. 236.

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Electrochemical Detection:

Couple the reaction with appropriate (enzymatic) reactions to produce an electrochemically detectable product.

References:

Inhibition and Molecular Structure:

1. Clonis, Y.D., M.J. Goldfinch, and C.R. Lowe, "The Interaction of Yeast Hexo Kinase EC-2.7.1.1 with Procion Green H-4G," Biochem. J., 197(1):203-212, 1981.

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Immobilization:

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2. Fuller, C.W., and H.J. Bright, "Covalent Immobilization of Soluble and Enzymically Active Adenine Nucleotide Coenzymes by a Single Step Procedure," J. Biol. Chem., 252(19):6631-6639, 1977.

3. Gacesa, P., and W.J. Whish, "A Novel Method of Coenzyme Immobilization," Biochem. Soc. Trans., 5(5):1407-1408, 1977.

4. Gray, D.N., and M.H. Keyes, "Immobilized Enzymes for Chemical Analysis," Chemtech., 7(10):642-648, 1977.

5. Kiang, S.W., J.W. Kuan, S.S. Kuan, and G.G. Guibault, "An Immobilized Hexokinase Enzyme Stirrer for a Simple and Economical Assay of Plasma Glucose," Clin. Chim. Acta., 78(3):495-498, 1977.

6. Leon, L.P., A.R. De Noto, L.R. Snyder, and C. Horvath, "Co-Immobilized Hexo Kinase and Glucose 6 Phosphate Dehydrogenase from the Determination of Serum Glucose on Technicons Continuous Flow Systems," Clin. Chem., 22(7):1223, 1976.

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8. Miura, Y., K. Miyamoto, H. Urabe, H. Tanaka, and T. Yasuda, "Reduction in Apparent Michaelis Constant Values of Enzymes of ATP by Immobilization on an Anion Exchanger," J. Ferment Technol., 57(5):440-444, 1979.

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9. Yamazaki, Y., H. Maeda, and H. Suzuki, "Preparation of N-6-N-6 Aminohexylcarbamoyl Adenine Nucleotides and Their Application to Coenzymically Active Immobilized ADP and ATP and Affinity Adsorbents," Eur. J. Biochem., 77(3):511-520, 1977.

IV. Phosphorylase EC-2.4.1.1

Reaction Catalyzed: Glycogen breakdown
$(\alpha\text{-}1,4\text{-glucosyl})_n + P_i = (\alpha\text{-}1,4\text{-glucosyl})_{n-1}$ + glucose-1-phosphate

Supplier: Sigma Chemical (P 6635, Phosphorylase b)

Biological Source: Rabbit muscle

Stability: Estimated 6 mos. at 5°C.

Activity: Approximately 30 IU/mg

Cost: $10.00/10mg

Reported Inhibition by Aromatic Chlorinated Hydrocarbons:

Polychlorinated biphenyls and polybrominated biphenyls, p-chlorophenol

Assay Procedure:

Illingsworth, B., and G.T. Cori, Biochem. Prep., 3:1-9, 1953.

Electrochemical Detection:

Couple the reaction with appropriate (enzymatic) reactions to produce an electrochemically detectable product.

References:

Inhibition and Molecular Structure:

1. Anderson, R.A., and D.J. Graves, "Chemistry of the Adenosine Monophosphate Site of Rabbit Muscle Glycogen Phosphorylase: I. Hydrophobic Nature and Affinity Labeling of the Allosteric Sites," Biochemistry, 12:1895-1900, 1973.

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4. Fujita, T., J. Iwasa, and C. Hansch, "A New Constant, χ, Derived from Partition Coefficients," J. Am. Chem. Soc., 86:5175-5180, 1964.

5. Graves, D.J., and J.H. Wang, "α-Glucan Phosphorylases -- Chemical and Physical Basis of Catalysis and Regulation," ed. P. Boyer, Enzymes, (3d ed), 7:435-482, 1972.

6. Mead, R.C. et.al., "Inhibition of Purified Rabbit Muscle Phosphorylase a and Phosphorylase b by Polychlorinated Biphenyls, Polychlorinated Biphenylols, and Polybrominated Biphenyls," Biochem. Biophys. Acta., 701:173-179, 1982.

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8. Soman, G. and G. Philip, "The Nature of the Binding Site for Aromatic Compounds in Glycogen Phosphorylase b," Biochim. J., 147:369-371, 1975.

9. Weber, I.T., L.N. Johnson, K.S. Wilson, D.G.R. Yeates, D.L. Wild, and J.A. Jenkins, "Crystallographic Studies on the Activity of Glycogen Phosphorylase b," Nature, 274:433-437, 1978.

V. ATPase EC-3.6.1.3

Reaction Catalyzed: ATP + H_2O = ADP + inorganic phosphate

Supplier: Sigma Chemical (A 7510 ATPase Grade IV)

Biological Source: Pork brain

Stability: Estimated 1 yr. at 5°C.

Activity: 0.3 IU/mg

Cost: $8.00/5U

Reported Inhibition by Aromatic Chlorinated Hydrocarbons:

Aldrin, dieldrin, DDT, chlorinated ethers, chlordane, PCB's

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Assay Procedure:

Cantley, L.C., Jr. and L. Josephson, Biochem., 15:5280-5287, 1976.

Electrochemical Detection:

Couple the reaction with appropriate (enzymatic) reactions to produce an electrochemically detectable product.

References:

Inhibition and Molecular Structure:

1. Bandyopadhyay, S.K., R.K. Tiwari, A. Bhattacharyya, and G.C. Chatterjee, "Effect of Dieldrin on Rat Liver Plasma Membrane Enzymes," Toxicol. Lett., 11(1-2):131-134, 1982.

2. Bansal, S.K., and D. Desaiah, "Effects of Substrate and Cations on the Inhibitory Action of Chlordecone on Rat Brain Synaptosomal Sodium Potassium ATPase," J. Miss. Acad. Sci., 26(Suppl.):53, 1981.

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APPENDIX B

PROCEDURES

Lactate Dehydrogenase Assay Procedure

The Worthington Enzyme Manual (1) was used as the initial source for a procedure for determining enzyme activity. Phosphate buffer was chosen as the enzyme buffer since it stabilizes the enzyme's physical structure (2). The final concentrations in the reaction cuvette were as follows: 1 mM sodium pyruvate, 220 μM NADH, 0.1 M potassium phosphate buffer, pH 7.3. 0.02 units LDH was added, and OD_{340} was recorded for 10 min. The enzyme activity is expressed as:

$$\text{Units/mg} = \frac{A_{340}/\text{min}}{6.2 \times \text{mg enzyme/ml reaction mixture}}$$

1. Decker, L.A., Worthington Enzyme Manual, Worthington Biochemical Corporation, Freehold, New Jersey, 1977, pp. 19-22.

2. Lovell, S.J. and D.J. Winzor, "Effects of Phosphate on the Dissociation and Enzymic Stability of Rabbit Muscle Lactate Dehydrogenase," Biochem. 13:17, 1974.

Lactate Dehydrogenase Inhibition Assay Method

Reaction Catalyzed: pyruvate + NADH + H^+ ⟶ lactate + NAD^+

Reagents:

Lactate Dehydrogenase (1.1.1.27): Type M_4 Isoenzyme (rabbit muscle), Sigma Chemical Company No. L5132, Lot No. 50F-9716 (1387 units/ml). For a standard solution, make a 1:693 dilution of the enzyme, 2 units/ml buffer - 20% methanol (.0027 mg/ml).
Sodium Pyruvate: 10 mM (in buffer)
NADH: 2.2 mM (in buffer)
Inhibitor: 10^{-3} M, 10^{-4} M, 10^{-5} M (in methanol)
Buffer: 0.1 M potassium phosphate buffer pH 7.3
Methanol Buffer: 0.1 M potassium phosphate buffer-14.7% methanol, adjusted to pH 7.3 with HCl.

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Reaction Conditions (in the cuvette):

Lactate Dehydrogenase: 0.1 unit/ml (135 ng/ml)
Sodium Pyruvate: 1 mM
NADH: 220 μM
Inhibitor: 10^{-4} M, 10^{-5} M, 10^{-6} M
Potassium Phosphate Buffer: 0.09 M pH 7.3
Methanol: 20.6%

Procedure:

1. Set the Hitachi 100-80 recording spectrophotometer at 340 nm.

2. Add, as follows, to the sample cuvette:

Buffer-14.7% methanol	0.65 ml
Sodium Pyruvate	0.10 ml
NADH	0.10 ml
Inhibitor or methanol blank	0.10 ml

3. Mix the contents and incubate 1 min. in the spectrophotometer sample compartment.

4. Add 50 μl enzyme to the cuvette and record OD_{340} for 10 min.

△pH Detection Assay

Reagents:

LDH: 4 units/ml (in buffer)
NADH: 2.2 mM (in buffer)
Pyruvate: 10 mM (in buffer)
Buffer: 10^{-3} M acetate buffer - 20% methanol; pH initial = 6.0.
Adjust pH to 7 with 1.2×10^{-4} M $[OH^-]$.

Reaction Conditions:

LDH: 0.4 units/ml (540 ng/ml)
Pyruvate: 1 mM
NADH: 220 μM
Acetate Buffer: 10^{-3} M; pH 7.0
Methanol: 20%

Procedure:

1. Add, as follows, to a 20 ml beaker:

acetate-methanol buffer	7 ml
NADH	1 ml
LDH	1 ml

2. Mix, measure pH.

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3. Add 1 ml pyruvate, mix. Record ΔpH for 5 minutes with the Beckman Model 76 pH meter.

Immobilized LDH Assay

Standard conditions are as previously reported.

0.1 M potassium phosphate buffer pH 7.4
220 μM NADH
1 mM sodium pyruvate
20% methanol

Procedure:

1. Record initial Abs_{340} of the above reaction mixture.

2. Mix 3 ml of the above with a measured amount of LDH gel in a syringe equipped with a glass wool filter plug and stirrer flea.

3. Expel aliquots at timed intervals for recording of Abs_{340}.

4. Return aliquot to syringe after each recording.

5. Repeat steps 1-3 with increasing amounts of LDH gel until the observed kinetics approach V_{max} conditions.

When testing inhibitors, incorporate inhibitor concentrate in the above standard reaction mixture.

LDH Immobilization Method Polyacrylamide Gel Entrapment Procedure

Reagents:

Acrylamide: 7.3 M (in buffer)
N,N-Methylene-bis-Acrylamide: 0.15 M (in buffer)
N,N,N',N'-Tetramethylethylenediamine (TEMED)
Potasssium Persulfate: .163 M (in water)
Phosphate-Methanol Buffer: 0.1 M potassium phosphate buffer-10% methanol; pH = 7.4
LDH: 735 units/mg protein

Final Conditions:

Volume: 2 ml
Acrylamide: 0.94 M
N,N-Methylene-bis-Acrylamide: 0.1 M
$K_2S_2O_8$: 6.5×10^{-3} M
TEMED: .08%
LDH: 0.5 mg

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Procedure:

1. Combine reagents in 3 ml syringe. Mix.
2. Deaerate and gel 30 minutes under vacuum in dessicator.
3. Force gel through #20 gauge needle.
4. Work gel particles into a fine paste using a pestel and #40 mesh stainless steel screen.
5. Wash the dispersed gel 5x in buffer.
6. Collect the washed gel in a syringe fitted with a glass wool filter plug to retain gel. Expel excess buffer.
7. Test buffer for "free enzyme".

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